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MARGINAL BANDS OF LOBSTER BLOOD CELLS: DISAPPEARANCE ASSOCIATED WITH CHANGES IN CELL MORPHOLOGY

WILLIAM D. COHEN, IRIS NEMHAUSER, AND MARION F. COHEN

Department of Biological Sciences, Hunter College, 695 Park Ave., New York, NY 10021, and The Marine Biological Laboratory, Woods Hole, MA 02543.

Abstract

Blood cells of the lobster *Homarus americanus* were examined for the presence of marginal bands of microtubules (MBs*). Nearly all of the cells were highly flattened and elliptical immediately after removal from the animals, and they contained cytoplasmic granules. Rapid cell lysis with Triton X-100 under microtubule-stabilizing conditions produced granule-free "clots" in which almost every nucleus had an associated MB. Thin sections of intact, oriented cells revealed MBs of 50–60 microtubules in classical location in the plane of cell flattening, close to (but not touching) the plasma membrane. Cells rapidly lost native morphology as they spread on glass substrata. The fate of the MB during spreading was followed in lysed cell "cytoskeletons" under phase contrast and by means of indirect anti-tubulin immunofluorescence. MBs initially appeared twisted, then splayed apart, and finally disappeared completely after cell spreading for 20 min. These observations and previous literature support identification of the MB-containing cells as the clotting cells of *Homarus*, in which the *in vitro* behavior may reflect a self-destructive *in vivo* mechanism. Their similarity to clotting cells of other species is discussed.

Introduction

The comparative hematology of invertebrates has received relatively little attention since the publication of Andrew's text (1965), but the recent comprehensive survey by Ratcliffe and Rowley (1981) has helped to renew interest in the fascinating array of invertebrate blood cell types. It is evident, however, that the study of invertebrate blood cells is somewhat inhibited by the confusing terminology with which they are described. While they are often referred to in general as "hemocytes" which exist in "hemolymph", their specific classification has been made by various authors on different bases such as staining properties ("basophils", etc.), morphological features ("granulocytes", etc.), motile behavior ("amebocytes"), and anatomical source ("coelomocytes"). Additional complexity arises from the tendency of certain cell types to undergo rapid changes of morphology after removal from the animal. Thus a population of cells which are initially fairly uniform in shape may exhibit a diversity of shapes in cytological preparations.

These problems are quite apparent in studies of the blood cells of the lobster, *Homarus americanus*. Three papers describing the hemocyte types in this species (Toney, 1958; Hearing and Vernick, 1967; Cornick and Stewart, 1978) differ with respect to the basis of their specific classification, and thus their data are difficult to correlate. Moreover, *Homarus* hemocytes undergo dramatic shape changes sub-

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* Abbreviations: MB = marginal band, EGTA = ethyleneglycol-bis (β-aminoethyl ether) N, N′-tetra acetic acid, TAME = p-tosyl arginine methyl ester HCl, PIPES = piperazine-N-N′-bis (2-ethane sulfonic acid)

sequent to removal from the animal. Most of the cells are initially flattened and elliptical, but rapidly lose their ellipticity and assume various shapes while spreading thinly on substrata (Toney, 1958).

With respect to their initial flattened, elliptical morphology, lobster hemocytes are similar to all non-mammalian vertebrate erythrocytes (Andrew, 1965) and to *Limulus* amebocytes (Armstrong, 1979), all of which contain marginal bands of microtubules (MBs) as part of their cytoskeletal system (Fawcett and Witebsky, 1964; Goniakowska-Witalinska and Witalinski, 1976; Nemhauser *et al.*, 1980). The purpose of this study was to determine whether lobster blood cells also contain MBs, and, if so, whether alterations of MB structure accompany *in vitro* changes in cell morphology.

Materials and Methods

Lobsters (*Homarus americanus*) were maintained in running sea water or in cooled (15°C), aerated tanks of "Instant Ocean" artificial sea water (Aquarium Systems, Inc.). Blood (hemolymph) was obtained from the animal by rapidly snipping a walking leg and drawing the exuded fluid into a Pasteur pipette. Fresh samples were used for each experiment. When required, marine crustacean Ringer's solution, pH 7.0 (Cavanaugh, 1975), was used as a blood diluent. Cell morphology was examined via phase contrast microscopy of both living cells and cells fixed in Ringer's containing glutaraldehyde (1% or 0.1% equally effective). Fixation was particularly valuable since it prevented changes of morphology during handling and observation of the material.

For thin sectioning, fresh blood was immediately diluted approximately 1:10 into fixative consisting of 9 volumes marine crustacean Ringer's solution and 1 volume 10% glutaraldehyde. Samples were then placed onto polylysine-coated plastic coverslips (Bel-Art Inc.). The coverslips had been pre-treated for 15 min with a 1% solution of polylysine of MW > 400,000, followed by water washes and drying. The samples were incubated on these coverslips in a moist chamber for 1 h at room temp., during which time many of the fixed cells adhered to the polylysine substrate with their plane of flattening approximately parallel to that of the coverslip. Following washes in Ringer's and in 0.1 *M* phosphate buffer, pH 6.9, the adhering material was post-fixed in 1% OsO_4 in the same buffer for 1 h. The coverslips, with adhering material, were then washed in phosphate buffer, dehydrated in an ethanol series, infiltrated with Epon-ethanol (to avoid possible dissolution of the plastic coverslip in propylene oxide), and flat-embedded in Epon. The coverslips were subsequently peeled away from the Epon, and some embedments were glued flat onto the tips of Epon capsule blanks for sectioning approximately parallel to the plane of cell flattening. Other embedments were recoated with Epon to sandwich the cells, and oriented such that sections were cut approximately perpendicular to the plane of cell flattening. Orientation of the cells was surveyed under phase contrast by means of 2 μm thick sections. Thin sections were cut with a diamond knife on the MT-2 ultramicrotome (DuPont-Sorvall Instruments), stained with saturated uranyl acetate in 50% ethanol followed by Reynold's lead citrate, and examined in the Hitachi HS-8 transmission electron microscope operating at 50 kV.

For rapid and convenient testing for the presence of MBs, cells were lysed with Triton X-100 under microtubule-stabilizing conditions. The lytic medium consisted of 1 m*M* $MgCl_2$, 5 m*M* EGTA*, 10 m*M* TAME, 100 m*M* PIPES, 0.4% Triton X-100, pH 6.8 with KOH. This medium (="LyM") has been employed previously to reveal MBs in both vertebrate and invertebrate blood cells (Cohen, 1978; Nemhauser *et al.*, 1980).

Indirect immunofluorescence after anti-tubulin binding was also used to visualize MBs during cell spreading. Fresh blood was immediately diluted approximately 1:10 into Ringer's and "zero-time" cytoskeletons prepared by 1:10 lysis in LyM. These cytoskeletons were found to adhere to glass coverslips which had been pre-cleaned with acid, after about 10 min incubation. Living cells were allowed to attach and spread on coverslips at room temperature (approx. 22°C) for 5, 10, and 20 min, during which time they were kept covered with Ringer's. To prevent the possible accumulation of secreted material during spreading, the coverslips were placed periodically in beakers with large volumes of Ringer's solution. At the appropriate times, the coverslips were drained briefly and the cells lysed by flooding with LyM. They were then rinsed with LyM lacking Triton and TAME, and fixed for 20 min in the same medium containing 3.7% (w/w) formaldehyde. After washing in the same medium without formaldehyde, followed by 3 washes in phosphate-buffered saline (PBS), the material was incubated with anti-tubulin for 30 min at 37°C. It was then rinsed again with PBS and similarly incubated with fluorescein-labelled goat anti-rabbit IGG. After washing in PBS, the coverslips were mounted in PBS for observation. The anti-tubulin was a gift of Dr. R. D. Goldman, then of Carnegie Mellon University. Anti-tubulin specificity had been verified by double immunodiffusion against 3×-cycled mammalian brain tubulin, by the ^{125}I-protein A binding procedure against α and β tubulin, and by using preimmune serum as a control (R. D. Goldman, personal communication). Zeiss phase contrast microscopes equipped with epifluorescence illumination were used throughout. Images were recorded on Plus-X or Tri-X film.

Results

When fresh blood samples were examined immediately in phase contrast or subsequent to dilution into Ringer's containing glutaraldehyde, the predominant cell type observed was highly flattened and elliptical (Fig. 1a). Ellipticity varied from slight (*i.e.* nearly circular, as in Fig. 1b) to extensive (elongate), with the long axis of the ellipse typically in the range of 15–20 μm. Cells of other morphology were present in lesser numbers, as illustrated in Figure 2. Counts were made of different cell types in random fields for a sample from one animal. Of 100 cells counted, there were 71 granular elliptical cells (category A) and 9 with one end oval and the other tapered and refractile (category B). Careful observation of cells in flow under the coverslip showed that the latter cell type was identical to the former except that one end was twisted relative to the other, and thus this morphological type represented about 80% of the population. Some of the remaining cells had one or two sharply pointed ends (category C), and others were generally elliptical in outline but had bulges or constrictions (category D). Many of the celi shapes in these minority categories (Fig. 2) appeared to be variations on a basic flattened elliptical form, so that the 80% figure is probably minimal. Most of these cells contained conspicuous granules which ranged from about 0.3 to 1 μm in diameter, as illustrated in Figure 1c.

Rapid dilution of blood samples with lysing medium containing Triton X-100 (LyM) directly upon removal from the animal produced masses or "clots" of lysed cells. Such clots did not contain any of the cytoplasmic granules visible in the intact cells; nuclei were the most conspicuous components (Fig. 3a). At higher magnification nearly all of the nuclei were found to be circumscribed by phase-dense MBs (Fig. 3b). The bands are only about 0.2–0.4 μm thick; careful focussing was, therefore, required to reveal their presence about nuclei at different levels within the "clot". If the material was squashed under the coverslip, many of the MBs became

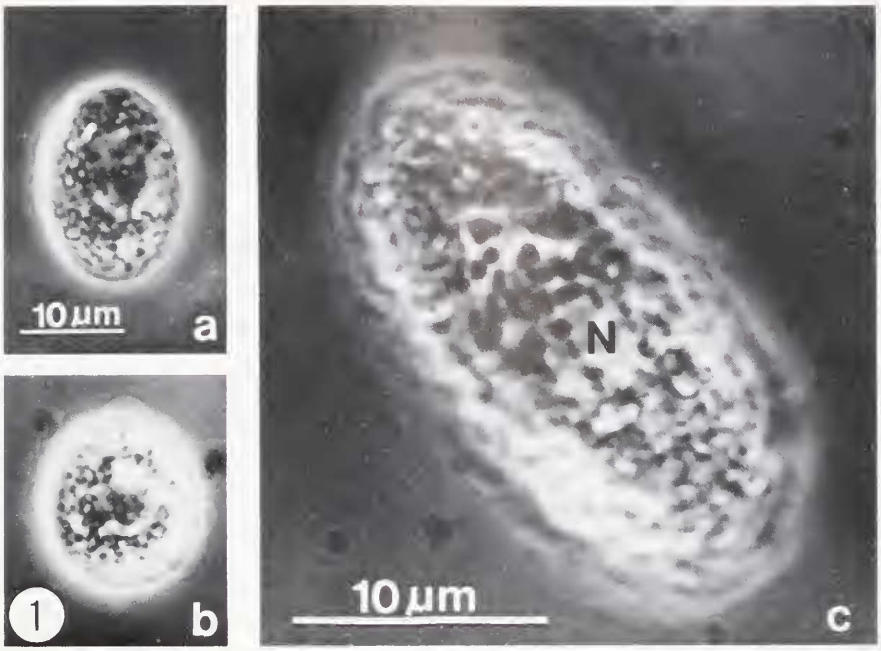

FIGURE 1. *Homarus* blood cells fixed immediately after removal from the animal (in crustacean Ringer's solution containing 1% glutaraldehyde). Nearly all cells were highly flattened; most were elliptical as in (a), but some were more circular (b). The great majority of cells contained conspicuous granules, about 0.3 to 1 µm in diameter. These were most readily visible under oil immersion by focussing in the thinner cytoplasmic layer above or below the nucleus (N), as illustrated in (c). Phase contrast; mag. bar for (b) as in (a).

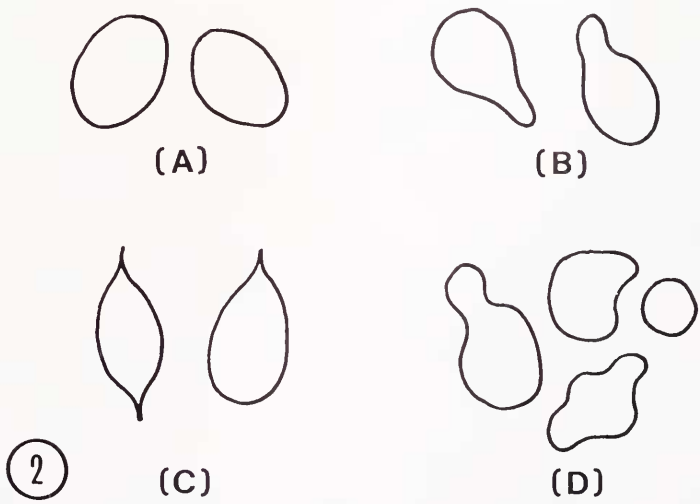

FIGURE 2. Morphology of blood cells fixed immediately after removal from the animal (fixation: 1:10 dilution into Crustacean Ringer's solution containing 1% glutaraldehyde). One hundred cells in non-selected fields were surveyed in phase contrast; the number (= percentage) of cells in each category is given in parenthesis. Morphology category A: cells flattened and elliptical (71); category B: one end flattened and elliptical, other end rounded, tapered, and refractile (9); category C: flattened, elliptical, with a sharp point at one or both ends (6); category D: various other shapes (14).

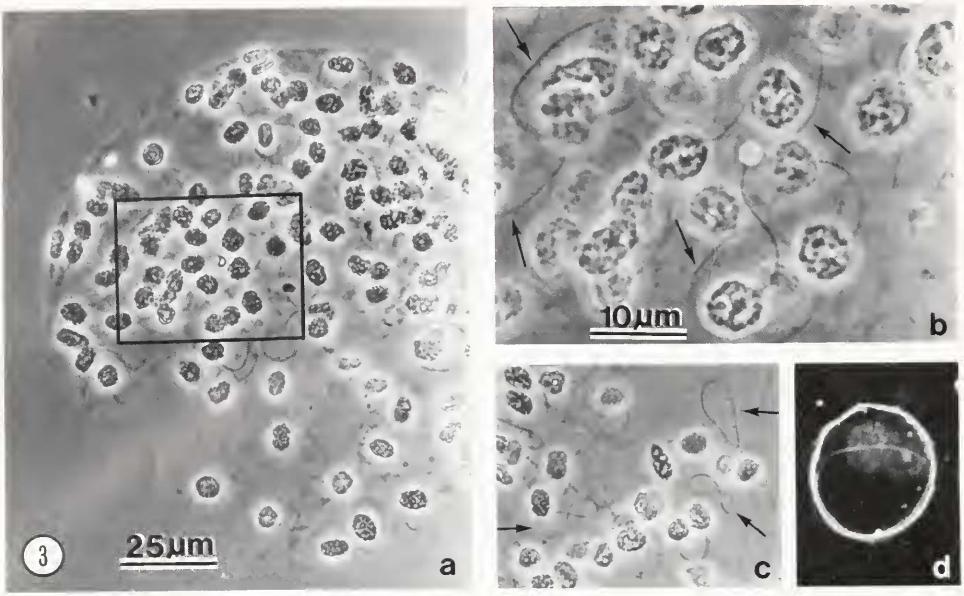

FIGURE 3. Marginal bands of lobster blood cells visualized by direct, rapid dilution of blood sample with lytic medium containing Triton X-100 (LyM). (a) "Clot" of lysed cells produced by 1:10 blood dilution into LyM, in which nuclei are prominent and granules are not present. (b) Higher magnification view of area delimited by the rectangle in (a), showing MBs adjacent to individual nuclei (arrows). (c) Squashed "clot" showing some MBs free of nuclei (arrows). (d) MB of lysed cell as visualized by indirect anti-tubulin immunofluorescence. MB microtubules are brightly fluorescent. In most cells fluorescence was confined to the MB, with a separated microtubule or thin bundle occasionally present, as illustrated. (a–c) Phase contrast; mag. bar for (c) as in (a). (d) Fluorescence microscopy, bar for (d) as in (b).

displaced from nuclei, appearing as free closed loops or figure-8 forms (Fig. 3c). When fresh blood samples were first rapidly diluted 1:10 into crustacean Ringer's solution and then immediately diluted 1:10 again into LyM, large clots did not form, and there were many more individual lysed cells present. These also contained a nucleus circumscribed by the MB, with no visible granules. In preparations treated with anti-tubulin and examined by the indirect immunofluorescence technique, MBs were brightly fluorescent, with few or no microtubules visible except those of the MB bundle (Fig. 3d).

The generality of MB occurrence was examined with respect to lobster size and sex. As shown in Table I, MBs were present in hemocytes of both males and females 12 to 22 cm in length. The impression that nearly all cells contained MBs was verified by counts in non-selected fields which showed that approximately 95% of the nuclei were circumscribed by MBs.

In order to verify the expected location of the MBs in intact cells, and to demonstrate their microtubular substructure directly, cells were thin-sectioned either approximately parallel to or perpendicular to their plane of flattening. In the former case, elliptical or circular cell profiles were generally observed (Fig. 4a, b), most of which displayed prominent marginal bundles of microtubules in longitudinal or somewhat oblique section (Fig. 4c, d). When sections were perpendicular to the plane of flattening, cell profiles were relatively narrow with tapered ends (Fig. 5a), and the cross-sectioned bundle of MB microtubules was present in classical location at opposite ends near the cell surface (Fig. 5b, c). The MB contained about 60–70

TABLE I

Percentage of lobster blood cells containing marginal bands (MBs)[a]

Animal No. and Sex	Length[b] (cm)	Lysed cell counts (20 cells per animal)	
		Nuclei with MBs	Nuclei without MBs
1. ♂	12	20	0
2. ♀	16	20	0
3. ♂	17	19	1
4. ♂	18	18	2
5. ♀	20	19	1
6. ♂	22	18	2
	Totals . .	114	6
	Percentage (of 120) . .	95%	5%

[a] Procedure: sample of hemolymph from snipped leg was diluted approximately 1:20 into marine Crustacean Ringer's solution. The diluted sample was lysed by dilution approx. 1:10 into LyM (immediately!).

[b] Measured from tip of rostrum to tip of uropod.

microtubules, closely but somewhat irregularly packed. It was evident that MB microtubules were not in direct contact with the plasma membrane bilayer at their closest approach, but were separated from it by a layer of material approximately 50 nm thick (Fig. 5b).

Having demonstrated that MBs were present, the question was raised as to the fate of these MBs during morphological transformation of the cells *in vitro*. As observed in phase contrast, most cells were still relatively flat and elliptical one minute after removal from the animal, and in contact with glass (Fig. 6a). Within 5 min the cells became "spiky" and began to spread (Fig. 6b), and after 10 min most cells had spread so extensively that internal organelles began to become visible (Fig. 6c). Granules could be seen disintegrating in such spread cells. At this point, lysis by perfusion with LyM revealed no MBs (Fig. 7a, b).

In order to visualize the MB disorganization sequence, time-course samples were examined by means of anti-tubulin binding and indirect immunofluorescence. Major stages in the sequence are illustrated in Figure 8. At t = 0 (no spreading) the lysed cells contained essentially all of their microtubules within intact circular or elliptical MBs as shown in Figure 3d. After spreading on glass for 5 min, most of the MBs were still recognizable as such. Some were still elliptical (Fig. 8a), but most of them showed signs of disorganization, with figure-8 forms more common than usual (Fig. 8b). After 10 min, recognizable MBs were no longer present, and bundles of microtubules were splayed in various directions (Fig. 8c). By 20 min the microtubule network had disappeared completely, leaving only scattered points of fluorescence visible in some cells (Fig. 8d).

DISCUSSION

Presence of MBs in invertebrate blood cells

With respect to cytoskeletal structure and the presence of MBs, apparently diverse cell types in phylogenetically distant species share a major common feature. There are well-documented reports of MBs in blood cells of Echinoderms (sea cu-

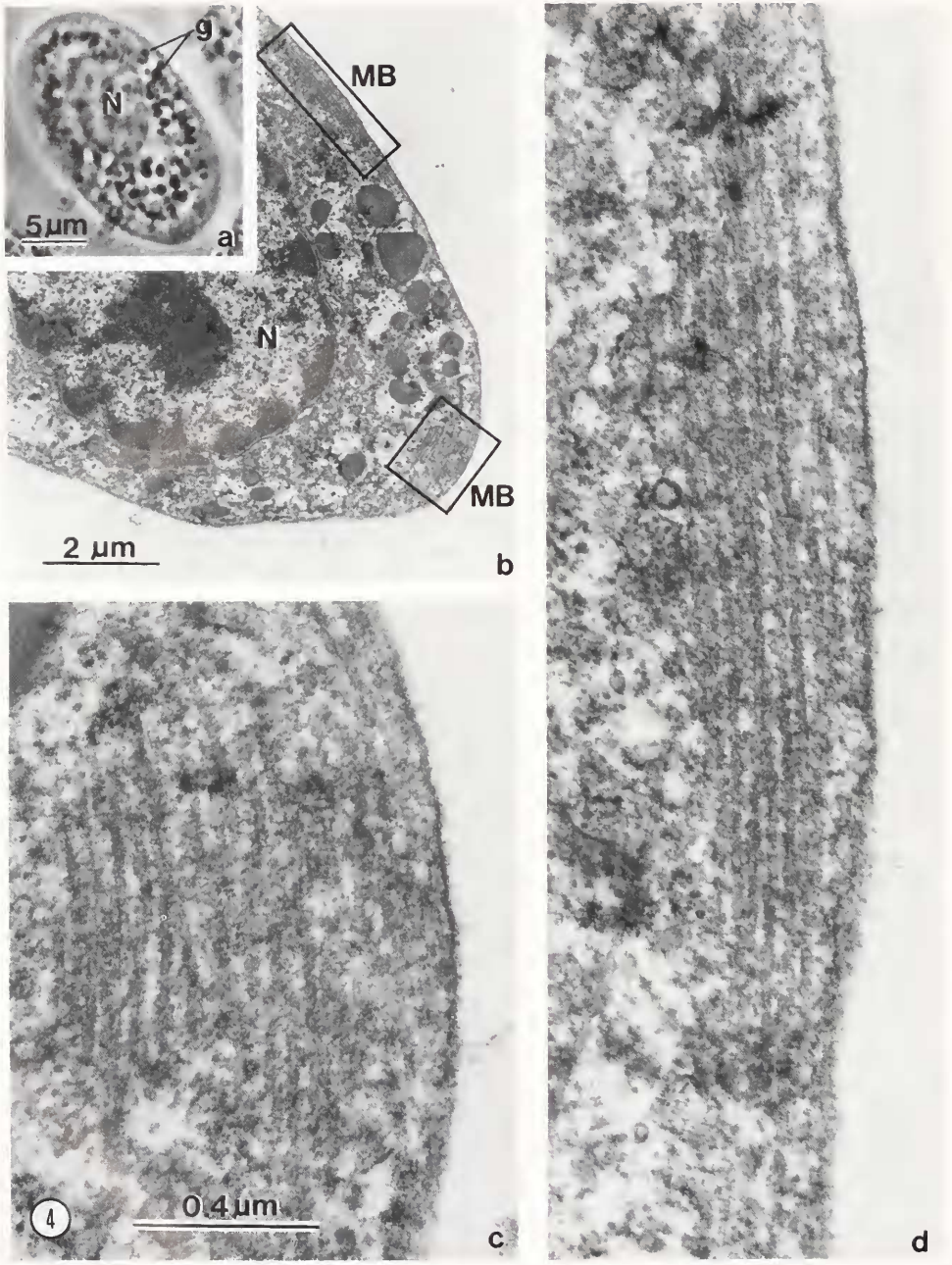

FIGURE 4. *Homarus* blood cells sectioned approximately parallel to the plane of flattening. (a) Thick section (2 μm) in phase contrast, showing general elliptical cell profile with nucleus (N) and granules (g) visible. (b) Transmission electron microscope survey view of part of a thin section comparable to the thick section in (a), with nucleus (N) and cytoplasmic granules visible. The MB appears in two locations at the periphery, as marked by the rectangles. (c, d) Lower and upper rectangles, respectively, of (b), at higher magnification. The MB microtubules are present in longitudinal view, running approximately parallel to the cell surface. Mag. bar for (d) as in (c).

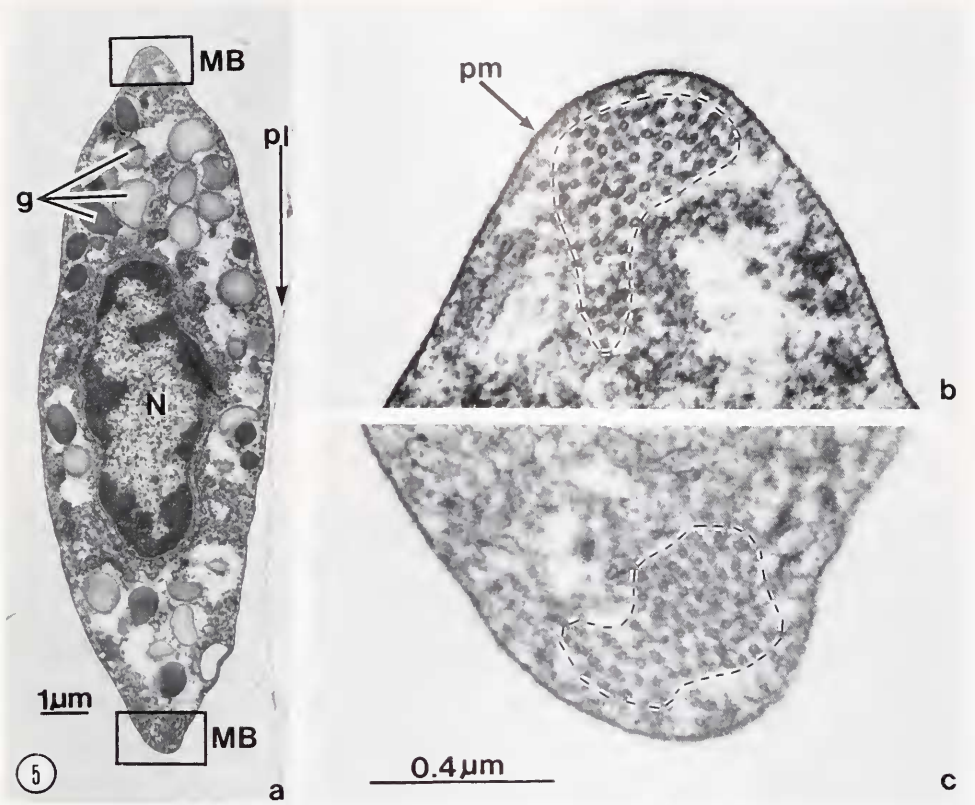

FIGURE 5. *Homarus* blood cell sectioned approximately perpendicular to the plane of flattening (transmission electron microscopy). (a) Complete section of cell, with prominent nucleus (N) and granules (g). The cell lies against the polylysine-coated substratum visible as a vertical dense line (arrow labelled pl). The MB is present in near cross-section in classical location at opposite tapered ends of the cell. (b, c) Higher magnification views of regions delimited by upper and lower rectangles, respectively, of (a). The bundle of MB microtubules is outlined by the dotted line, with 60–70 microtubules present. In (b), where the microtubules are in near cross-section, it is clear that the MB bundle does not directly contact the plasma membrane bilayer (pm), but is minimally separated from it by about 50 nm. Mag. bar for (c) as in (b).

cumber erythrocytes, Fontaine and Lambert, 1972), Sipunculans (*Phascolopsis gouldii* erythrocytes, Nemhauser *et al.*, 1980), Arthropods (cockroach hemocytes, Baerwold and Boush, 1970; *Limulus* amebocytes, Nemhauser *et al.*, 1980; lobster hemocytes, this paper), and Mollusks (erythrocytes of *Anadara* and *Noetia* "blood clam" species, Cohen and Nemhauser, 1980). These invertebrate phyla may thus be added to the vertebrates as represented by all non-mammalian vertebrate erythrocytes, "primitive" mammalian yolk-sac erythrocytes, and mammalian platelets (Fawcett and Witebsky, 1964; Behnke, 1965; van Deurs and Behnke, 1965), illustrating the fact that MBs are a nearly universal (though not necessarily constant) blood cell feature. Although it is possible that MBs may serve different functions in different cell types, it is likely that they share common functions in cellular morphogenesis (Barrett and Dawson, 1974) and in the adaptation of single cells to existence under flow conditions (Cohen *et al.*, 1982).

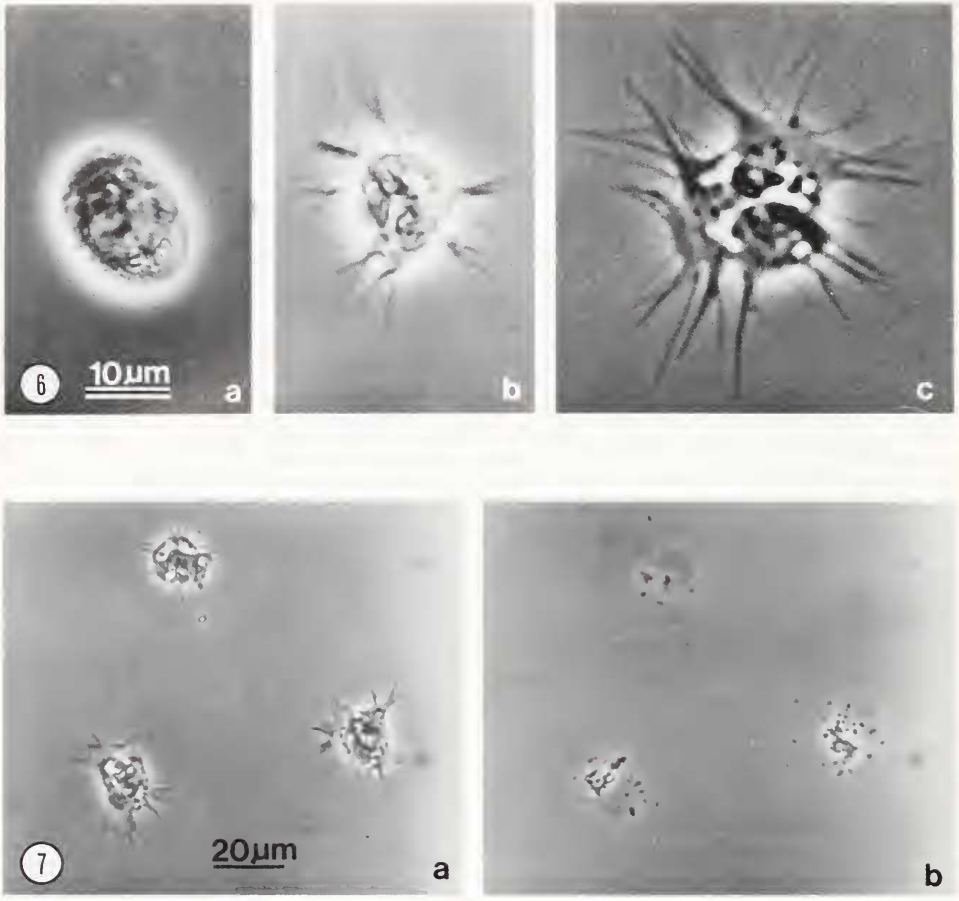

FIGURE 6. Typical spreading sequence for living cells in contact with glass substratum (phase contrast). (a) One min after removal from animal, in contact with glass; cell still elliptical and flat. (b) Same cell after 5 min, becoming amorphous and "spiky". (c) Same cell after 10 min, showing extensive spreading such that internal organelles start to become visible. The three photographs are at the same magnification, with mag. bars as in (a).

FIGURE 7. Three cells allowed to spread for 12 min, before (a) and after (b) lysis by perfusion with LyM. MBs are not present in such spread cells. Phase contrast, mag. bar for (b) as in (a).

Lobster blood cell types

According to Toney (1958), fresh lobster blood (*H. americanus*) observed in phase contrast contains four cell types: relatively small "lymphoid cells" (averaging 11×13 μm) and "monocytes" (7×10.8 μm), and larger "explosive refractile granulocytes" (16.8×25.2 μm) containing either small (.25 μm) or large (1 μm) granules. Nearly all of the cells were round or oval immediately after removal from the animal. The "explosive refractile granulocytes" were identified as active participants in blood clotting, with those containing large granules constituting 90–95% of the total blood cell population. These cells showed extensive morphological changes with time, spreading very thinly, with disintegration of the large granules at their surface producing an "explosive" or "bubbling" effect. Some cells ultimately became almost unrecognizable, with only the nucleus intact. "Explosive refractile granulocytes"

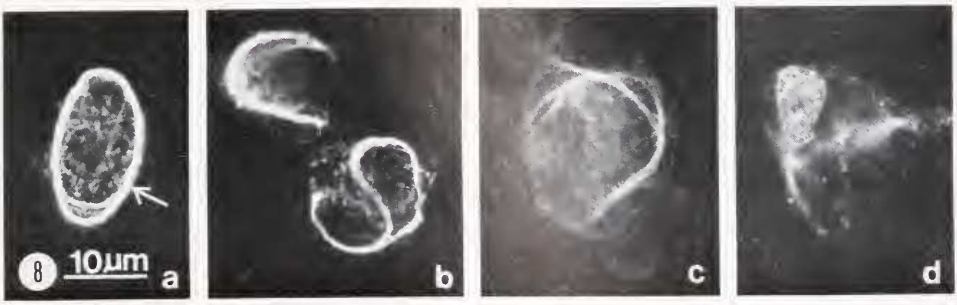

FIGURE 8. Fate of the MB during spreading as visualized by indirect immunofluorescence after anti-tubulin binding. (a, b) Five min spreading; MBs still recognizable as such. A few cells contain elliptical MBs (a, arrow), but in the majority the MBs show signs of disorganization, such as extensive twisting (b). (c) Ten min spreading; MBs are no longer recognizable. Microtubule bundles are evident, and there is a general background fluorescence. (d) Twenty min spreading; there are points of fluorescence visible, but microtubules are not present. The nucleus is also sometimes fluorescent, as in this example; it is not clear whether this is an artifact or may have some significance. Fluorescence microscopy, mag. bars as in (a).

were also described as being the major cell type in the blood of the crayfish (*Cambarus bartoni*) and the blue crab (*Callinectes sapidus*).

Hearing and Vernick (1967) examined the blood cells of *H. americanus* by light and electron microscopy, using samples fixed immediately after removal from the animal. Neither MBs nor microtubules were observed, and apparently they were not preserved by the fixation methods employed. Three cell types were identified and counted with respect to percentage of the population: "Eosinophils" (20% of population), "ovoid basophils" (20%), and "spindular basophils" (60%). All of the cells contained granules, with the largest ones observed in the "spindular basophils". Identification of cell type was based primarily upon the examination of cells in sections, and it appears that the plane of sectioning may not have been taken into account in interpreting the images. Thus it is likely that the "spindular basophils" were flattened, oval cells cut at various angles (as in Fig. 5), and that the "ovoid basophils" were the same cells in face view (Fig. 4). This cell type would then constitute about 80% of the population and contain the larger granules, justifying their identification as the "explosive granulocytes" of Toney (1958) and as the major cell type observed in the present work.

Using stained and unstained films of fixed cells, Cornick and Stewart (1978) described the hemolymph of *H. americanus* as containing four cell types: prohyalocytes (about 2%), hyalocytes (64%), eosinophilic granulocytes (12%), and chromophobic granulocytes (22%). Cells in the last three categories, comprising most of the population, were observed to contain granules of various sizes and staining properties; presumably, most of these cells also correspond to the "explosive granulocytes" of Toney (1958).

It is evident that the phenomenon occurring in the "explosive granulocytes" of the lobster is exocytosis, and that these cells are quite comparable morphologically to *Limulus* amebocytes (Levin and Bang, 1964a, b; Armstrong, 1979, 1980; Ornberg and Reese, 1981). In *Limulus*, all of the clotting factors including clottable protein are believed to be present in the amebocyte cytoplasmic granules (Murer *et al.*, 1975). In *Homarus*, however, fibrinogen has been reported to be a normal component of hemolymph plasma (Stewart *et al.*, 1966), and therefore the granules of *Homarus* blood cells are probably not identical in content to those of *Limulus*.

Homarus hemocyte extracts do initiate clotting (Stewart *et al.*, 1966), and presumably clotting factors are contained in the granules and released during exocytosis. Lysis of *Homarus* granules by Triton in the present work could thus account for the immediate formation of "clots" and the absence of granules from the lysed cells (Fig. 3). As the terminology is confusing with respect to such cells in the Crustacea and for invertebrates in general, we suggest that cells of this type be referred to collectively as "clotting cells". Preliminary observations indicate that MBs are present in similar cells in several species of marine crabs (Cohen *et al.*, 1977), and thus they may be a common feature of Crustacean clotting cells.

MB function

The lobster blood cell MB is a bundle of 60–70 microtubules which is present in classical location near the cell surface in the plane of cell flattening (Figs. 4, 5). As viewed in cross section, the MB microtubules do not make direct surface contact with the plasma membrane bilayer, but are separated from it by a layer of material associated with the cell surface (Fig. 5). Granules observed in thin sections also did not appear to be in direct contact with the bilayer. Gaps between granules and the plasma membrane bilayer have been well-documented in *Limulus* amebocytes prior to activation of exocytosis (Ornberg and Reese, 1981), and the MB microtubules of this species are similarly separated from the plasma membrane bilayer by a space, as seen in thin sections (Nemhauser *et al.*, 1980). These observations suggest that, at least prior to activation, the MB of both *Homarus* and *Limulus* blood cells is in contact with a surface-associated or cortical cytoskeletal layer which is continuous throughout the cell. Interaction between the MB and a sub-surface cytoskeletal layer has been proposed as the basis for MB influence on cell shape in non-mammalian vertebrate erythrocytes (Cohen, 1978; Cohen *et al.*, 1982), and may thus apply to the invertebrate clotting cells as well. If such a surface-associated cytoskeletal layer exists, passage through it would be a major step in secretory granule exocytosis in these cells (Ornberg and Reese, 1981).

MB disorganization during cell spreading

As visualized by indirect anti-tubulin immunofluorescence, lobster clotting cell MBs become disorganized during cell spreading and ultimately disappear. Although microtubule depolymerization and repolymerization may play a role, the images obtained give the impression that the MB initially twists and then splays apart. Splaying or stripping off of microtubules from the major MB bundle also appears to occur in human platelets during spreading, as seen in "spiral ring" forms visualized by anti-tubulin binding. At later stages platelet MBs are not recognizable, but microtubules are present in the cytoplasm and in pseudopodia (Debus *et al.*, 1981). Microtubule disappearance in the case of lobster clotting cells *in vitro* may reflect a more complete process of cellular self-destruction which is part of the *in vivo* lobster clotting mechanism.

Acknowledgments

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